A Proposal To Transfer *Microbispora bispora* (Lechevalier 1965) to a New Genus, *Thermobispora* gen. nov., as *Thermobispora bispora* comb. nov.

YUE WANG,* ZHENSHUI ZHANG, AND JISHENG RUAN

Microbial Collection and Screen Laboratory, Institute of Molecular and Cell Biology,
National University of Singapore, Singapore 119260

We determined almost complete 16S rRNA gene sequences of two *Microbispora bispora* (Lechevalier 1965) strains, ATCC 19993^T (T = type strain) and JCM 3082. The two sequences were 99% similar to each other but exhibited only 81 to 87.8% similarity with the 16S rRNA gene sequences of seven other *Microbispora* strains. A phylogenetic analysis revealed that the two sequences clustered not only distantly from other *Microbispora* strains, but also outside the cluster containing members of the family *Streptosporangiaceae*. On the basis of the results of our phylogenetic analysis and the results of a comprehensive review of the genus *Microbispora* by Miyadoh et al. (S. Miyadoh, S. Amano, H. Tohyama, and T. Shomura, J. Gen. Microbiol. 136:1905–1913, 1990) in which chemotaxonomic and DNA-DNA hybridization analyses were performed, we propose that *Microbispora bispora* should be transferred to a new genus, *Thermobispora* gen. nov., as *Thermobispora bispora* comb. nov.

The genus *Microbispora* was proposed by Nonomura and Ohara (14) in 1957 for actinomycetes that form conspicuous aerial mycelia bearing longitudinal pairs of spores. This genus contains 11 validly described species, and *Microbispora rosea* is the type species (9, 10, 13–15). In 1990 Miyadoh et al. (12) conducted a comprehensive taxonomic review of the genus *Microbispora* by using chemotaxonomic and DNA-DNA hybridization techniques. On the basis of a mean DNA homology value of 56.6%, 10 *Microbispora* species were combined into one species, *Microbispora rosea*. *Microbispora bispora* (Lechevalier 1965) (9), which was originally described by Henssen as *Thermopolyspora bispora* (4), remained separate species mainly because of its low levels of DNA-DNA homology (mean, 16.4%) with the 10 other species.

There is evidence that *Microbispora bispora* strains differ in many ways from the other 10 *Microbispora* species. Some of the differences seem greater than the differences usually observed between different species belonging to the same genus. In addition to its low levels of DNA homology with the other *Microbispora* species, *Microbispora bispora* can also be distinguished on the basis of its growth temperature, its predominant menaquinone species, its phospholipid species, its major fatty acid species, and the electrophoretic pattern of its ribosomal proteins (12, 16). *Microbispora bispora* grows at 65°C but not at 35°C or lower temperatures. The 10 other *Microbispora* species can be divided into two groups, a mesophilic group and a thermophilic group, on the basis of their growth temperature ranges, but none of them can grow at temperatures above 60°C (4, 12). *Microbispora bispora* strains contain small amounts of glucosamine-containing phospholipids, and MK-9(H₀) is the predominant menaquinone of this organism, while the 10 other *Microbispora* species contain large amounts of glucosamine-containing phospholipids and MK-9(H₄), MK-9(H₂), and MK-9(H₀) are the predominant menaquinones (12). Ochi et al. reported that *Microbispora bispora* ATCC 19993^T (T = type strain) produced a ribosomal protein pattern

on a two-dimensional polyacrylamide gel electrophoresis (PAGE) analysis gel that was very different from the patterns produced by other *Microbispora* strains (16). Furthermore, *Microbispora bispora*, *Microtetraspora pusilla*, and *Microtetraspora ferruginea* were found to have comparable levels of DNA-DNA homology with the 10 other *Microbispora* species (12).

16S rRNA gene sequence-based phylogenetic analysis has proven to be a powerful tool for determining relationships between organisms that more conventional methods have problems defining (18, 21–23). In the present study, we determined the 16S rRNA gene sequences of two *Microbispora bispora* strains (strains ATCC 19993^T and JCM 3082) and analyzed the phylogenetic relationships between these strains and other species or strains belonging to the genus *Microbispora* and members of other actinomycete groups.

MATERIALS AND METHODS

Organisms and culture conditions. *Microbispora bispora* JCM 3082, *Kutzneria viridigrisea* JCM 3282^T, and *Actinomadura madurae* JCM 7436^T were purchased from the Japan Collection of Microorganisms, Wako, Japan. *Microbispora bispora* ATCC 19993^T and ATCC 15737, *Actinoplanes philippinensis* ATCC 12427^T, *Dactylosporangium aurantiacum* ATCC 23491^T, *Micromonospora chalybeata* ATCC 12452, and *Micromonospora echinospora* subsp. *echinospora* ATCC 15837^T were obtained from the American Type Culture Collection, Rockville, Md. *Actinoplanes auranticolor* IFO 12245^T, *Microbispora bispora* IFO 14880^T, and *Dactylosporangium roseum* IFO 14352^T were obtained from the Institute for Fermentation, Osaka, Japan. *Microbispora bispora* DSM 43038 was obtained from DSM-German Collection of Microorganisms and Cell Cultures. The organisms were cultured in the media and conditions described by the suppliers.

Culture observations and morphological characterization. *Microbispora bispora* ATCC 19993^T and JCM 3082 were inoculated onto International *Streptomyces* Project medium 4 agar, Bennett's agar, and IFO328 agar (Institute for Fermentation). After inoculation, sterilized glass coverslips were inserted in the agar media. The plates were incubated at 28, 37, and 60°C for 14 days. The coverslips were stained and examined with a light microscope (model DM RB; Leitz).

Preparation of genomic DNA. Mycelia of actinomycete strains were harvested by centrifugation at 3,600 × g for 5 min in a model 5410 C microcentrifuge (Eppendorf, Hamburg, Germany). Approximately 200 µl of wet mycelia was transferred to a 1.5-ml Eppendorf microcentrifuge tube. The mycelia were resuspended in 500 µl 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 each tube was incubated at 65°C for 30 min. Each 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

* Corresponding author. Mailing address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260. Phone: 65 7783207. Fax: 65 7791117. Electronic mail address: mcbwangy@leonis.nus.sg.

centrifuged in an Eppendorf microcentrifuge at 14,000 × g for 5 min. The supernatant was decanted, and the tube was left inverted on a piece of paper towel for 10 min. The nucleic acid was resuspended in 100 µl of 10 mM Tris–1 mM EDTA (pH 7.5) containing 100 µg of DNase-free RNase A (Sigma Chemical Co.) per ml and incubated at 37°C for 30 min. 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'TACCTGAT AGCGGCCGAGAGTTTGATCCTGGCTCAG3' (nucleotides 8 to 27 of the *Escherichia coli* 16S rRNA gene) (1) and 5'TACAGGATCCGCGGCCGTAC GG(C/T)TACCTGTTTACGACTT3' (nucleotides 1492 to 1513) (the underlined sequences are cleavage sites of restriction enzyme *NorI*). The nonspecific nucleotides on the 5' side of the *NorI* site were included for efficient cleavage by *NorI*. The ends of cloned 16S ribosomal DNAs were sequenced by using the M13 forward sequencing primer 5'GTITTTCCAGTACGAC3' and reverse primer 5'GCGGATAACAATTTCACACAG3'. The internal regions were sequenced in both orientations by using the following eight oligonucleotide primers whose target sequences were obtained either from previous reports (8) or from an alignment of the 16S rRNA gene sequences of many actinomycete species: 5'GCCTATCAGCTTGTGGT3' (nucleotides 239 to 256), 5'CGTGCCAGCA GCCGCGGT3' (nucleotides 514 to 531), 5'TAGATACCTGGTAGTCC3' (nucleotides 789 to 806), 5'CCGCAACGAGCGCAACCC3' (nucleotides 1097 to 1114), 5'TACCGCGGC(G/T)GCTGGCAC3' (nucleotides 532 to 515), 5'G GACTACC(A/C/G)GGGTATCTAAT3' (nucleotides 806 to 787), 5'GGGTGTCGCTCGTTGCGG3' (nucleotides 1114 to 1097), and 5'ACGGGCGGTGTG TAC (nucleotides 1406 to 1392). All of the oligonucleotides were synthesized by Oligos Etc., Inc., Wilsonville, Ore.

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 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 2.5 U of *Taq* DNA polymerase (Amersham, Aylesbury, Buckinghamshire, England), and 1× buffer (the buffer provided with *Taq* DNA polymerase); the final volume was 100 µl. The PCR program used 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 BRL GIBCO FastCheck nucleic acid quantification system (GIBCO BRL Life Technologies, Gaithersburg, Md.).

Cloning and sequencing of 16S ribosomal DNA. The purified PCR products of 16S rRNA genes were cut with *NorI* (Amersham) and cloned into plasmid Bluescript SK (Stratagene, La Jolla, Calif.). *E. coli* DH5αF cells (GIBCO BRL) were prepared and transformed as described previously (11). Recombinant clones were picked and grown in 2 ml of Luria-Bertani (11) medium overnight, and the recombinant plasmid DNA was prepared by using a miniplasmid preparation kit (QIAGEN, Germany). Each cloned 16S ribosomal DNA 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 of the DNASTAR program (DNASTAR, Inc., Madison, Wis.). Phylogenetic trees were constructed by using the maximum-parsimony method contained in the PAUP package (19). The sequences were added randomly to circumvent the problem of entrapment in local optima. The confidence level of the phylogenetic tree topology was evaluated by performing 100 bootstrap replications with the BOOTSTRAP program contained in the same package.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under the following accession numbers: *Microbispora bispora* JCM 3082, U58524; *Microbispora bispora* ATCC 19993^T, U58523; *Actinoplanes philippinensis*, U58525; *Actinoplanes auranticolor*, U58526; *Actinomadura madurae*, U58527; *Dactylosporangium aurantiacum*, U58528; *Dactylosporangium roseum*, U58529; *Kutzneria viridogrisea*, U58530; *Micromonospora calcea*, U58531; and *Micromonospora echinospora* subsp. *echinospora*, U58532.

The GenBank accession numbers of 16S rRNA gene sequences that we reported previously (20) are as follows: *Actinomadura atramentaria*, U49000; *Actinomadura citrea*, U49001; *Actinomadura coerulea*, U49002; *Actinomadura cremea* subsp. *rifamycinii*, U49003; *Actinomadura echinospora*, U49004; *Actinomadura fulvescens*, U49005; *Actinomadura kijaniata*, U49006; *Actinomadura libanotica*, U49007; *Actinomadura luteofluorescens*, U49008; *Actinomadura macra*, U49009; *Actinomadura rugatobispora*, U49010; *Actinomadura verrucosopora*, U49011; *Microbispora aerata*, U48984; *Microbispora amethystogenes*, U48988; *Microbispora chromogenes*, U48989; *Microbispora diastatica*, U48990; *Microbispora parva*, U48985; *Microbispora thermodiastatica*, U14986; *Microbispora thermorosea*, U48987; *Microtetraspora africana*, U48842; *Microtetraspora angiospora*, U48843; *Microtetraspora fastidiosa*, U48844; *Microtetraspora ferruginea*, U48845; *Microtetraspora fusca*, U48973; *Microtetraspora glauca*, U48974; *Microtetraspora helveta*, U48975; *Microtetraspora niveoalba*, U48976; *Microtetraspora polychroma*, U48977; *Microtetraspora pusilla*, U48978; *Microtetraspora rectitena*, U48979; *Microtetraspora roseola*, U48980; *Microtetraspora salmonea*, U48982; *Microtetraspora spiralis*, U48983; *Streptosporangium corrugatum*, U48991; *Streptosporangium fragile*, U48992; *Streptosporangium longisporum*,

U48993; *Streptosporangium nondiastaticum*, U48994; *Streptosporangium pseudovulgare*, U48995; *Streptosporangium roseum*, U48996; *Streptosporangium violaceochromogenes*, U48997; *Streptosporangium viridialbum*, U48998; and *Streptosporangium vulgare*, U48999.

The sequences of the following organisms were retrieved from public databases: *Actinosynnema mirum* (EMBL accession number X84447), *Bifidobacterium bifidum* (GenBank accession number U25952), *Geodermatophilus obscurus* (EMBL accession number L40620), *Kibdelosporangium aridum* (EMBL accession number M29283), *Nocardia asteroides* (EMBL accession number X57949), *Nocardia seriolae* (EMBL accession number X36925), *Promicromonospora enterophila* (EMBL accession number X83807), *Saccharothrix coeruleofusca* (EMBL accession number X76963), *Saccharothrix mutabilis* (EMBL accession number X76965), *Saccharopolyspora erythraea* (EMBL accession number X53198), *Saccharopolyspora hirsuta* (EMBL accession number X53196), *Saccharomonospora caesia* (EMBL accession number Z38024), *Saccharomonospora viridis* (EMBL accession number Z38007), *Streptomyces coelicolor* (EMBL accession number Y00411), *Streptomyces galbus* (EMBL accession number X79852), *Tsukamurella inchenonensis* (EMBL accession number X85955), and *Tsukamurella paurometabola* (EMBL accession number Z36933).

RESULTS AND DISCUSSION

Cultural and morphological features. The cultural and morphological features of *Microbispora bispora* strains are generally the same as those described previously (4, 9). These bacteria are gram-positive organisms which form substrate mycelia whose hyphae are 0.5 to 0.8 µm in diameter and aerial mycelia that branch monopodially and bear longitudinal pairs of spores. The spore diameters are usually 1.2 to 2.0 µm, but in liquid media spores with a diameter of 3 µm have been observed. The aerial mycelia are white, and the substrate mycelia are yellow or yellowish brown on the media which we used. No soluble pigment is produced.

Comparative 16S rRNA gene sequence analysis. We determined almost complete 16S rRNA gene sequences (nucleotides 3 to 1491 [*E. coli* numbering] [1]) for two *Microbispora bispora* strains, strains JCM 3082 and ATCC 19993^T. We also determined the 16S rRNA gene sequences of *Microbispora bispora* ATCC 15737, DSM 43038 (equivalent to JCM 3082), and IFO 14880 (equivalent to ATCC 19993^T) in order to confirm the reliability of our sequence results. A pairwise sequence similarity analysis revealed that these five sequences were 99 to 99.8% similar. In the analysis described below only the sequences of two strains, strains ATCC 19993^T and JCM 3082, were included (these sequences have been deposited in the GenBank database). Table 1 shows that the sequences of strains ATCC 19993^T and JCM 3082 are 99% similar to each other, but only 81 to 87.8% similar to the 16S rRNA gene sequences of other *Microbispora* strains (belonging to 7 of the 10 *Microbispora* species combined in the taxon *Microbispora rosea* by Miyadoh et al. [12]), which are 95.8 to 99.2% similar to each other. *Microbispora bispora* also exhibits low levels of sequence similarity (about 81%) to *Microtetraspora glauca* and *Streptosporangium roseum*, the type species of two other genera in the family *Streptosporangiaceae* (3); in contrast, the average levels of similarity between the latter two species and the seven other *Microbispora* strains studied are 95 and 91%, respectively. We noticed that the levels of similarity between the sequence of *Microbispora bispora* and the sequences of *Microtetraspora glauca* and *Streptosporangium roseum* (about 81%) are much lower than the intergeneric levels of similarity in the family *Streptosporangiaceae*, which range from 90 to 96% (20). The 81% value is even lower than the levels of similarity between the *Actinomadura* species of the *Thermomonosporaceae* and members of the *Streptosporangiaceae* (>88%) (20). These observations demonstrate that *Microbispora bispora* is only distantly related to other *Microbispora* strains and other members of the family *Streptosporangiaceae*.

To determine the phylogenetic affiliation between *Microbispora bispora* and members of the family *Streptosporangi-*

% Similarity

[illegible]

FIG. 1. Alignment of 16S rRNA gene sequences. The 16S rRNA gene sequences of two *Microbispora bispora* strains were aligned with the sequences of members of other genera of the family Streptosporangiaceae. The nucleotide sequence of *Actinomadura madurae* 16S rRNA gene was also included. The region corresponding to *E. coli* nucleotide positions 57 to 243 (1) is shown. The nucleotides that differ from other nucleotides at the same position are enclosed in boxes. The multisequence alignment was prepared by using the MEGALIGN program of DNASTAR.

aceae, the sequence of *Microbispora bispora* was analyzed together with the sequences of 14 *Microtetraspora* species, 7 *Microbispora* strains, and 8 *Streptosporangium* species by using Swofford's PAUP (phylogenetic analysis using parsimony) program (19). The sequences of 12 *Actinomadura* species were also included in the analysis because of the historical taxonomic association of these organisms with members of the Streptosporangiaceae (2, 3, 7). All of the sequences could be aligned without generating significant ambiguity, except that the two *Microbispora bispora* sequences contained some nucleotides which were not present in the other sequences, including the sequences of the seven other *Microbispora* strains. Figure 1 shows the region corresponding to nucleotides 57 to 243 of the *E. coli* 16S rRNA gene (1). The sequences of the *Microbispora bispora* strains had a stretch of 11 nucleotides around position 210 which seemed to be unique to this species because it was not found in any other actinomycete 16S rRNA genes retrieved from the public databases. Extra nucleotides were also observed in the region around nucleotide 80, and these nucleotides were not present in the sequences of the other members of the Streptosporangiaceae. These nucleotide signatures could serve as markers for identification of *Microbispora bispora* strains. Inclusion or exclusion of these nucleotides in the phylogenetic analysis had no effect on the topology of the most parsimony tree shown in Fig. 2a. As we have reported elsewhere (20), the seven strains of *Microbispora rosea* formed a major cluster with the members of the genera *Microtetraspora* and *Streptosporangium*, and all of the *Actinomadura* species formed a second major cluster; the stabilities of these two major clusters were supported by 99 and 100 bootstrap values, respectively. The two *Microbispora bispora* strains were located some distance outside the two major clusters representing the families Streptosporangiaceae and Thermomonosporaceae (3, 7). To determine the possible affiliation of *Microbispora bispora* with other groups of actinomycetes, we compared the *Microbispora bispora* sequences with the sequences of 39 representative species belonging to 19 genera in a phylogenetic analysis. The phylogenetic tree which we obtained is shown in Fig. 2b. Again, *Microbispora bispora* was located outside the clusters containing *Actinomadura* species and the members of the Streptosporangiaceae. However, the *Microbispora bispora* strains appeared to be most closely related to the members of the latter two groups, forming a cluster of a higher order with a significant bootstrap value (94). The results of the sequence

similarity and phylogenetic analyses described above demonstrate that *Microbispora bispora* does not belong to the genus *Microbispora* and very likely does not even belong to the family Streptosporangiaceae.

The phylogenetic separation of *Microbispora bispora* from the genus *Microbispora* may gain strong support from several distinctive properties of this organism. The absolutely thermophilic lifestyle of *Microbispora bispora* can be distinguished from the mesophilic and facultatively thermophilic (growth at both 35 and 55°C) lifestyles of the other *Microbispora* strains. It is reasonable to conclude that organisms that grow at temperatures as high as 65°C need proteins with very different thermostabilities than the thermostabilities of the proteins of organisms that grow at temperatures which are 10 to 30°C lower. This hypothesis is supported by the very different ribosomal protein pattern of *Microbispora bispora* revealed by a two-dimensional PAGE analysis (16) and by the low levels of DNA-DNA association with other *Microbispora* strains (12). *Microbispora bispora* can also be distinguished by chemotaxonomic properties. This organism contains MK-9(H₀) as the predominant menaquinone; in contrast, the predominant menaquinones found in other *Microbispora* strains are MK-9(H₄), MK-9(H₂), and MK-9(H₀) (12). *Microbispora bispora* also contains much lower amounts of glucosamine-containing phospholipid and 10-methyl fatty acid than other *Microbispora* strains (12).

Proposal to transfer *Microbispora bispora* to a new genus. On the basis of phylogenetic, chemotaxonomic, and DNA-DNA hybridization evidence and growth temperature data, we propose that *Microbispora bispora* (Lechevalier 1965) should be transferred to a new genus, *Thermobispora* gen. nov. The genus name refers to the thermophilic lifestyle of the organism and to the formation of pairs of spores on aerial hyphae. The type species is *Thermobispora bispora* comb. nov. However, the taxonomic position of the genus *Thermobispora* within the order Actinomycetales cannot be determined because the 16S rRNA gene sequences of members of many actinomycete genera are not available for comparison.

The taxonomic status of the 10 *Microbispora* strains combined by Miyadoh et al. as *Microbispora rosea* (12) on the basis of DNA-DNA association values needs to be reviewed. Obviously, when *Thermobispora bispora* was used as a reference, the levels of DNA-DNA relatedness among the 10 other species appeared to be higher. The new taxonomic position of *Thermobispora bispora* also explains the comparable levels of DNA-

FIG. 2. Phylogenetic trees for *Microbispora bispora* strains with members of the family Streptosporangiaceae and *Actinomadura* species (a) and with 39 representative species of 19 actinomycete genera (b). The trees were generated by using a maximum-parsimony method (19). Treatment of alignment gaps either as a NEWSTATE or as MISSING had little effect on the general topologies of the trees. The numbers at the nodes are bootstrap values (based on 100 resamplings). (a) Bar = 1 inferred substitution per 100 nucleotides. (b) Bar = 5 inferred substitutions per 100 nucleotides. Trees with similar topologies were also generated by using the Saitou-Nei (17) neighbor-joining method contained in the Clustal V multiple-sequence alignment package (5) (data not shown).

DNA homology (about 16%) of this organism and *Actinomadura* species to the other *Microbispora* strains (12). Furthermore, more than 60% of the previously reported levels of DNA homology among the 10 strains were less than 60% (12), which does not justify combining all 10 species into one taxon (6). The same concern was also raised in previous reports (16, 20).

Description of *Thermobispora* gen. nov. *Thermobispora* (Ther.mo.bi.spo'ra. Gr. adj. *thermos*, hot; Gr. adj. *bis*, two; Gr. fem. n. *spora*, seed; M. L. fem. n. *Thermobispora*, high temperature two-spored organisms). The strains are gram-positive, aerobic, non-acid-fast, thermophilic organisms that produce substrate mycelia with hyphae which are 0.5 to 0.8 μ m in diameter. The aerial mycelia branch monopodially and bear longitudinal pairs of spores which are usually arranged alternately on side branches arising from the main hyphae. The smooth-surface spores are oval to spherical and nonmotile. Fragmentation of mycelia is not observed on agar or in liquid media. Neither sclerotia, sporangia, nor any other special structure is formed. The cell wall contains a major amount of meso-diaminopimelic acid, and the whole-cell hydrolysate contains madurose and galactose. MK-9(H_0) is the major menaquinone. The phospholipid pattern type is type PIV, and the phospholipids include a small amount of glucosamine-containing phospholipids. 14-Methylpentadecanoic acid is the major fatty acid. The DNA G+C content is 70 mol%.

Description of *Thermobispora bispora* (Lechevalier 1965) comb. nov. *Thermobispora bispora* (bi. spo'ra. Gr. adj. *bis*, two; Gr. fem. n. *spora*, seed; M. L. fem. adj. *bispora*, two spores) type strain ATCC 19993 grows at 50 to 65°C but not at 35°C. This organism is an absolutely thermophilic actinomycete. Sub-

strate and aerial mycelia are formed. Longitudinal pairs of spores are borne on the branches of the aerial mycelia. The diameters of the spores are usually 1.2 to 2.0 μ m, but occasionally spores with a diameter of 3 μ m are observed in liquid culture. The aerial mycelia are white and the substrate mycelia are yellow or yellowish brown on International *Streptomyces* Project medium 4 and IFO328 medium. No soluble pigment is produced. Starch is not hydrolyzed. Negative for iodine production and nitrate reduction. Inositol and rhamnose are utilized for growth, but arabinose and glycerol are not utilized.

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