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A New Genus of the Actinomycetales: *Microtetraspora* gen.nov.

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SUMMARY

Two aerobic mesophilic microbial species of a new genus belonging to the family Streptomycetaceae of the order Actinomycetales are described under the name *Microtetraspora* (*Microtetraspora glauca*, type species). The micro-organisms produced a filamentous growth which is differentiated into a vegetative and an aerial mycelium.

The new genus is characterized by the formation of a short and sparsely branched aerial mycelium bearing at the end of short sporophores chains of four spores. Sporulation is not observed to take place on the vegetative mycelium. The genus *Microtetraspora* is widely distributed in the soil and has been isolated from many samples.

INTRODUCTION

The number of genera of the actinomycetes, or ray fungi, has increased considerably in the last few years reflecting a continued interest in these micro-organisms. Thus, while at the time of the latest edition of *Bergey's Manual* (1957) only three genera had been recognized in the Streptomycetaceae, by 1961 Waksman listed the following six genera: *Streptomyces*, *Thermoactinomyces*, *Microbispora* (Waksmania), *Thermopolyspora*, *Micromonospora* and *Thermomonospora*.

The present paper reports the isolation and description of two strains of actinomycetes which were easily distinguished from the known genera of the Streptomycetaceae by their unusual mode of sporulation: a chain of four spores was formed on short sporophores developing on monopodially branched short strands of aerial mycelium. The strains differed significantly from the previously described genera in the family Streptomycetaceae and it is therefore proposed to include them in a new genus to which the name *Microtetraspora* gen.nov. is given. The type species of the genus is *Microtetraspora glauca* (ATCC 23057) a subculture of which has been deposited with the American Type Culture Collection, together with *Microtetraspora fusca* (ATCC 23058) the second species isolated.

METHODS

A recently developed novel method for the mass isolation of little-known soil micro-organisms belonging to the Actinomycetales was used (Thiemann, in preparation). With this method the large-scale isolation of *Microbispora*, *Streptosporangium*, *Actinoplanes*, *Microtetraspora*, and species of other not yet described new genera has been made possible. A detailed description of this method will be published separately elsewhere.

Stains. The staining procedures recommended by the Society of American Bacteriologists (1957) were used.

Photographs. Microphotographs were taken with a Zeiss binocular microscope, model Standard WL, with Optovar and 35 mm. photomicrography attachment. Kodak Plus X Pan film was used. Electron photomicrographs of sporulated mycelium were taken with a Hitachi model HU-II electron microscope by touching collodion films mounted on grids onto cultures growing on soil agar incubated at 30°. Sporulation was studied also by growing the cultures directly on collodion membranes according to the technique described by Grein (1955).

Culture media. Unless otherwise stated the media used were prepared according to Waksman (1961).

Soil agar. Air-dried garden soil passed through a 10-mesh sieve, 30 g., agar 20 g., tap water to 1000 ml., pH 6·0. Sterilized for 20 min. at 120°.

Examination of spore germination. The spores from a fully sporulated agar slant culture were carefully scraped off with a sterile loop and streaked on to the surface of soil extract agar plates and soil agar plates. The plates were incubated at 28° and examined directly under the microscope for the appearance of germ tubes.

Preparation and analysis of cell walls. The organisms were grown in the seed medium of Margalith & Pagani (1961) and incubated at 28° on a rotary shaker (250 rev./min.). After 48 hr of growth the cells were collected by centrifugation and thoroughly washed with distilled water. After treatment with ethanolic KOH (0·5 %) at 37° for 24 hr the cells were washed with ethyl alcohol until the pH value was neutral. Suspensions of cells were disrupted by agitating them for 48–72 hr on a rotary shaker (250 rev./min.) with an equal volume of glass beads (1·0–1·5 mm. diam.). A few ml. chloroform were added to avoid bacterial contamination. The degree of breakage was checked by phase microscopy. For all the other steps the methods described by Becker, Lechevalier & Lechevalier (1965) were followed.

RESULTS

Description of Microtetraspora Thiemann, Pagani and Beretta gen.nov.

Morphology. The fine mycelium (about 1 μ diam.) differentiated into: (1) vegetative (primary) mycelium which was profusely branched, grew into the agar medium and formed a compact layer on top of it; (2) aerial (secondary) mycelium arising from the vegetative mycelium which grew into the air away from the agar surface. Spores were formed only on the aerial hyphae and never on the vegetative mycelium. The spores were formed in chains of four on short sporophores, which branched characteristically from the aerial hyphae at an angle of 45°, giving to the sporophores, when examined *en masse*, an appearance reminiscent of an ear of wheat (Pl. 1, fig. 1).

Description of Microtetraspora glauca ATCC 23057 and Microtetraspora fusca ATCC 23058 Thiemann, Pagani and Beretta, sp.nov.

Of the various cultures of *Microtetraspora* isolated, strain T 158 and strain T 457 were selected as the type strain of the species *M. glauca* and *M. fusca* respectively. No qualitative differences were found in the microscopic properties of *M. glauca* and *M. fusca*; the two isolates are, however, easily distinguished by their cultural and biochemical characteristics which are shown in Table 1 and Table 2 respectively. The following general morphological description applies equally to both cultures.

Vegetative mycelium. About 0·7–1·0 μ diam. Filaments long, wavy, branching, penetrating the agar and forming on its surface, compact, raised and tough colonies

like those of typical *Streptomyces* spp. The main (older) mycelium strands are larger (1·0 μ) and have a granulated appearance. The mycelium does not fragment even in old cultures.

Aerial mycelium. Approximately 1·0 μ in diam. The hyphae emerging from the vegetative mycelium are erect, sparsely branched and rather short, usually not exceed-

Table 1. *Cultural characteristics of Microtetraspora glauca strain T158 and M. fusca strain T457*

G. growth; V.m. vegetative mycelium; A.m. aerial mycelium; Sp. sporulation.
Soluble pigments were not formed on any of the media examined.
Colours were determined according to Maerz & Rea Paul (1950).

Culture media	*M. glauca* strain T158 (ATCC 23057)	*M. fusca* strain T457 (ATCC 23058)
Glucose asparagine agar	G. moderate, flat V.m. ash grey (pl. 27, A-2) A.m. sparse to absent Sp. absent	G. good, smooth V.m. hyaline A.m. white grey (pl. 11, A-1) Sp. good
Glycerol asparagine agar	G. poor, flat V.m. hyaline A.m. absent Sp. absent	G. good, smooth V.m. hyaline A.m. white grey (pl. 11, A-1) Sp. good
Calcium malate agar	G. very poor, flat V.m. hyaline A.m. sparse Sp. poor	G. poor, flat V.m. hyaline A.m. white grey (pl. 11, A-1) Sp. good
Hickey and Tresner agar	G. very good, elevated V.m. blue green (pl. 25, J-6) to yellowish green (pl. 21, J-1) A.m. abundant, pearl grey (pl. 44, A-1) Sp. good	G. very good, wrinkled V.m. brown violet (pl. 8, L-8) A.m. good, white grey (pl. 11, A-1) Sp. good
Soil agar	G. moderate, flat V.m. hyaline to ash grey (pl. 27, A-2) A.m. white grey (pl. 11, A-1) Sp. very good	G. moderate, flat A.m. white grey (pl. 11, A-1) Sp. very good
Bennett agar	G. very good V.m. blue green (pl. 26, K-5) to yellowish green (pl. 20, K-1) A.m. grey (pl. 28, C-1) Sp. moderate	G. very good, wrinkled V.m. amber with reddish tinge (pl. 12, B-9) A.m. white grey (pl. 11, A-1) Sp. very good
Oatmeal agar	G. good, flat V.m. blue green (pl. 30, G-1) A.m. blue grey (pl. 28, L-1) Sp. good	G. very poor, flat V.m. hyaline A.m. scarce to absent Sp. absent
Nutrient agar	G. moderate V.m. cream (pl. 9, D-2) A.m. absent Sp. absent	G. very good, elevated V.m. amber (pl. 12, F-7) A.m. absent Sp. absent
Carrot plug	G. no growth	G. very poor; wrinkled V.m. cream (pl. 9, D-2) A.m. absent
Potato plug	G. no growth	G. very poor; wrinkled V.m. cream (pl. 9, D-2) A.m. absent
Czapek agar	G. no growth	G. no growth

ing 50 μ. On rich media, such as Hickey and Tresner agar or even oatmeal agar, coremium-like formations of the aerial mycelium can be seen.

Sporophores. The sporophores are formed as thin (0·5 μ) monopodial branches of the aerial hyphae. The sporophores can be relatively long (5·5 μ) but normally are only 1·0–2·0 μ in length (Pl. 1, fig. 2). Occasionally the sporophores are extremely short, giving the impression that the spores are attached directly to the hypha. The sporophores of *M. fusca* tend to fuse into more or less globular masses of spores as the culture ages (Pl. 1, fig. 3).

Table 2. *Biochemical characteristics of Microtetraspora glauca strain T158 and M. fusca strain T457*

	M. glauca strain T158 (ATCC 23057)	*M. fusca strain* T457 (ATCC 23058)
Nitrate reduction	Reduced	Not reduced
Tyrosine agar	Not hydrolysed; no melanoid pigment	Not hydrolysed; no melanoid pigment
Skim milk agar	Casein slowly digested	Casein slowly digested
H_2S*	Positive	Positive
Gelatin	Completely hydrolysed	Not hydrolysed
Starch	Slight hydrolysis	Not hydrolysed
Litmus milk	No coagulation, no peptonization	No coagulation, no peptonization
Peptone iron agar	No melanoid pigment	No melanoid pigment

* Lead acetate strips, according to Küster & Williams (1964).

Spores. Spores are formed in chains of four. Mature spores are spherical (1·5 μ) to slightly oval (1·4 × 1·7 μ). No spines or hairs are present on the spore surface (Pl. 1, fig. 4). Immature spores are rather variable, being somewhat cuboid (0·8 × 0·8 μ) or even triangular, as a result of their mode of formation; the central ones tending to be compressed and cuboid and the ones at the extremities of the spore chain are sometimes triangular (Pl. 1, fig. 5). The immature spores when examined under the oil-immersion lens give the impression of being 'empty' and present a central highly refractile spot; fully developed and mature spores are uniformly dark, opaque, spherical to oval and usually without refractile spots (Pl. 1. fig. 6). Spores are formed only on the aerial hyphae and never on the vegetative mycelium. Occasionally in old cultures on soil agar plates, spores come in contact with the agar surface and germinate giving the impression that they are formed on the vegetative mycelium.

Spore formation. Spore formation begins by a terminal enlargement of the aerial hyphae, the cytoplasm of which condenses giving a bead-like appearance. In some preparations, transverse septa separating the spores can be seen (Pl. 2, fig. 7), in others the early separation of the spores is indicated only by an area of higher refringence. Four spores are usually formed in each chain but in some instances chains with only two or three spores are formed and very rarely chains of five spores. The four spores of a sporophore do not always grow uniformly and attain the same size; sometimes mature spores of variable size and immature ones occur in the same chain. Usually the four spores of the sporophore are formed in a single plane; however, occasionally the two spores at the top of the sporophore are placed eccentrically in relation to the other two. Sporulation in the genus *Microtetraspora* appears to proceed from the base of a fertile branch towards its tip, since transverse septa, when observed, were always seen to be formed first at the base of the fertile branch (Pl. 2, fig. 8). The spores when

transferred to fresh medium germinate and produce one to four rather large germ tubes. The germinated spores are clearly visible in the matted mycelium even 72 hr after germination (Pl. 2, fig. 9).

Staining. Young mycelium grown under submerged conditions was used for the staining reactions. The mycelium of *Microtetraspora glauca* and *M. fusca* was Gram-positive and was not acid-fast.

Effect of temperature and pH on growth. The effect of temperature on growth was investigated by incubating inoculated Petri dishes (Bennett agar, oatmeal agar and Hickey & Tresner agar) at 22°, 28°, 37°, 45°, and 50°. *Microtetraspora glauca* grew equally well on all three media at 22° and 37°, but was optimal at 28°, whereas *M. fusca* grew better at 28° and 37° than at 22°. Neither *M. glauca* nor *M. fusca* developed at 45° or 50°. To test for the effect of pH on growth, the above media were corrected to pH 4, 5, 6, 7 and 8, inoculated and incubated at 28°. Under these conditions *M. glauca* did not grow at pH 4; at pH 5 it was sparsely developed, whereas at pH 6, 7 and 8 good development was observed on all three media. *Microtetraspora fusca* on the other hand, grew equally well at pH 6 and 7, and only sparsely at pH 5 and 8. No growth took place at pH 4.

Utilization of carbon sources. Microtetraspora glauca and *M. fusca* did not develop on the standard minimal media employed in the carbon utilization tests. Positive results were obtained using the synthetic medium of Magni & von Borstel (1962) to which a vitamin B mixture (0·2 γ/ml) was added. Since on this medium the control plates (no carbon source) showed a moderate amount of growth as well, carbon utilization was considered negative when the growth was similar to or less than the growth on medium without a carbon source. Under these conditions, arabinose, xylose, glucose, galactose, fructose, mannose and ribose supported good growth of *M. glauca*, whereas rhamnose, maltose, saccharose, mannitol, inositol and starch supported only moderate to sparse growth. No growth was supported by lactose, raffinose, glycerol, sorbitol, dulcitol, inulin and sorbose. On the other hand *M. fusca* grew only in presence of arabinose, xylose, glucose, ribose, inulin and maltose. With all the other carbon sources, no visual difference between the control plates was observed.

Table 3. *Major components of cell-wall preparations of various form genera of Actinomycetales showing some morphological similarity with Microtetraspora*

	DAP acid		Sugars and amino-sugars			Amino acids	
Genera	LL	Meso	Galactose	Galactosamine	Arabinose	Glycine	Lysine
*Streptomyces**	+	−	−	−	−	+	−
*Microellobosporia**	+	−	−	−	−	+	−
*Microbispora**	−	+	−	−	−	(+)†	−
*Micropolyspora**	−	+	+	−	+	−	−
Microtetraspora	±	+	−	+	−	+	+

All genera had in common: muramic acid, glucosamine, glutamic acid and alanine.
* Data from Becker, Lechevalier & Lechevalier (1965).
† Only some strains do contain glycine.

Cell-wall composition. Chemical analysis of cell-wall preparations of *Microtetraspora* showed them to contain, as major components, meso-diaminopimelic acid, glucosamine, galactosamine, muramic acid, glycine, glutamic acid, alanine and lysine.

LL-diaminopimelic acid was always present, however, in small amounts. In Table 3 the cell-wall components of the various form-genera of the Actinomycetales showing some morphological similarity with *Microtetraspora* are given. As can be seen from this table, the cell walls of *Microtetraspora* differ considerably in their composition from the cell walls of the other genera described in the literature and with which *Microtetraspora* has some morphological affinity.

Source. Microtetraspora glauca was first isolated from a soil sample collected from the vicinity of carrot roots at Appiano Gentile, Como, Italy, and *M. fusca* from a sample received from Thailand and collected on a rubber plantation in the Province of Amphur Sadao, Songkhla. Species of *Microtetraspora* are, however, widespread in nature and have been isolated from many of the soil samples so far examined, received from various parts of Italy, Thailand and Brazil.

DISCUSSION

The growth characteristics on different nutrient media and the mode of spore formation indicate that the new genus *Microtetraspora* should be included in the family Streptomycetaceae. The characteristic production of a linear chain of four spores on the aerial mycelium differentiated these cultures from the previously described species that we have found in the literature and from those that we have isolated and studied up till now.

The genus *Micropolyspora* described by Lechevalier, Solotorovsky & McDurmont (1961) and characterized by the formation of chains of spores both on the aerial and on the vegetative mycelium differs from *Microtetraspora* not only in the formation of spores on the vegative mycelium but also in the overall morphology of the sporophores and aerial mycelium. Whereas in *Microtetraspora* as a rule, chains of 4 spores are formed, attached to the hypha by short sporophores, in *Micropolyspora* spore chains of up to 10 spores are not uncommon. Stolon-like structures formed by the long and branching hyphae of the aerial mycelium have also been described for *Micropolyspora* as well as for the genus *Microbispora*. Two new genera recently described having a short row of spores are *Microellobosporia* (Cross, Lechevalier & Lechevalier, 1963) and *Microechinospora* (Koniev, Tsyganov, Minbayev & Morozov, 1965). *Microellobosporia* which belong to the Actinoplanaceae is characterized by the production of large (1·5–3·5 μ) and non-motile sporangiospores arranged in a single straight row inside a sporangium formed both on the aerial and on the vegetative mycelium. The same genus was described simultaneously by Russian workers under the name *Macrospora* (Tsyganov, Zhukova & Timofeeva, 1963). The genus *Microechinospora* characterized by the formation on the aerial as well as on the vegetative mycelium of a straight row of large spherical spores (2·5–3·5 μ) enclosed in a club-shaped sporangium having a characteristically spiny surface is also included in the Actinoplanaceae. The genus *Microechinospora* closely resembles the genus *Microellobosporia* from which it is differentiated mainly by the spiny surface of the sporangia. The mode of sporulation, size of the spores, and sporangia formation clearly distinguish these genera from *Microtetraspora*. Thorough examination of stained and unstained preparations of *Microtetraspora* with the optical as well as electron microscope (Pl. 1, fig. 4; Pl. 2, fig. 10) failed to reveal any indication of sporangia formation.

Recently, Lechevalier & Lechevalier (1965) proposed a classification of the aerobic

actinomycetes based on their morphology as well as on the chemical composition of their cell walls. On the basis of their data these authors grouped the euactinomycetes as follows. Family Dermatophilaceae, one genus, *Dermatophilus*; Family Actinoplanaceae, 6 genera, *Actinoplanes*, *Ampullariella*, *Spirillospora*, *Streptosporangium*, *Amorphosporangium* and *Microellobosporia*; Family Streptomycetaceae, one genus, *Streptomyces*; Family Micromonosporaceae one genus, *Micromonospora*; Family Nocardiaceae Fam.nov., 6 genera, *Thermoactinomyces*, *Thermomonospora*, *Microbispora*, *Micropolyspora*, *Pseudonocardia* and *Nocardia*. In the genus *Streptomyces sensu lato* of Lechevalier & Lechevalier (1965) were included the various forms described as *Streptoverticillium*, *Chainia* and *Actinopycnidium*. The cell-wall preparations from the organisms belonging to this genus contained only glucosamine, muramic acid, alanine, glutamic acid, glycine and LL-diaminopimelic acid. In recent years the cell-wall composition of the actinomycetes has been frequently used as an aid in their classification. Whether the classical taxonomic approach of Waksman (1961) to the classification of the actinomycetes is to be preferred to the new and undoubtedly also more objective approach of Lechevalier & Lechevalier (1965) is beyond our scope. It suffices to mention, however, that when atypical *Streptomyces* and *Nocardia* are examined only for their morphological and cultural characters it is often entirely a matter of personal preference whether an isolate is placed in one genus or the other (Waksman, 1961). In these dubious cases, the chemical approach to the classification has already proved to be of great value (Becker, Lechevalier, Gordon, & Lechevalier 1964; Becker *et al.* 1965).

Analysis performed on cell-wall preparations of *Microtetraspora* brought to light some interesting data. The presence of small but reproducible amounts of LL-diaminopimelic acid, the presence of glycine, and the absence of characteristic sugars in the cell-wall hydrolysates of *Microtetraspora* is an interesting feature shared also by the cell walls of *Streptomyces* spp.

On the other hand, the composition of the cell walls of *Microtetraspora* shows also some similarities with the one of *Microbispora*; the presence of galactosamine and lysine, apart from the small amount of LL-diaminopimelic acid, distinguishes nevertheless the cell walls of these two genera. On the basis of these overall similarities it is possible to think of *Microtetraspora* as forming a link between the *Streptomyces* and *Microtetraspora*. Apart of this hypothetical phylogenetic relationship between *Microtetraspora* and some of the other genera of the Actinomycetales, it is important to bear in mind that in addition to the morphological features which distinguish *Microtetraspora* from the genera already described, it differs from them also in the cell-wall composition.

The following revision of the existing key to the family Streptomycetaceae *sensu* Waksman (1961) is proposed to accommodate *Microtetraspora*:

1. Aerial (secondary) mycelium formed
   a. Spores formed in chains mainly on the aerial mycelium, rarely also on the substrate mycelium (Becker, Lechevalier & Lechevalier, 1965).
      Streptomyces Waksman & Henrici
   b. Spores formed in chains, both on the aerial and substrate mycelium.
      Micropolyspora Lechevalier, Solotorovsky & McDurmont
   c. Spores formed in chains of four on short sporophores on the aerial mycelium only.
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d. Spores occurring singly, in pairs or in chains
  a 1. Mesophilic forms, spores in pairs on sporophores on the aerial mycelium and with rare exception also on the substrate mycelium (Becker, Lechevalier & Lechevalier, 1965). *Microbispora* Nonomura & Ohara
  b 1. Thermophilic forms:
    1. Spores single on sporophores both on the aerial and substrate mycelium. *Thermoactinomyces* Tsiklinsky
    2. Spores single on aerial mycelium only. *Thermomonospora* Henssen
2. Aerial (secondary) mycelium not produced.
  a. Spores occurring singly on short sporophores. *Micromonospora* Orskov

The genus *Thermopolyspora* (Henssen, 1957), of questionable validity (Krasilnikov, 1964; Becker *et al.* 1965; Lechevalier, 1965), has been omitted since its characterization was based on cultures contaminated with bacteria of unknown nature and her original cultures of *Thermopolyspora polyspora* are now dead, and according to Rule 24 g of the *International Code of Nomenclature of Bacteria and Viruses* (1958): 'A name of a taxon is illegitimate...if the characterization of the group was based upon an impure or mixed culture.' The new thermophilic genus *Actinobifida*, isolated by Krasilnikov & Agre (1964), has also been omitted. This genus is characterized by the formation of single spores borne on the tip of dichotomously branching sporophores arising from the hyphae of the aerial mycelium or from the surface of the colony. This genus was included by Krasilnikov & Agre (1964) in the family Micromonosporaceae, it is felt, however, that it would be more properly included among the thermophilic forms (*Thermoactinomyces, Thermomonospora*). Where *Actinobifida* will finally be placed has to await the results of more detailed comparative studies between *Actinobifida* and the other actinomycetes which form single isolated spores on the aerial mycelium.

Twelve soil samples out of a total of 53 different ones so far examined and received from various parts of the world yielded strains of *Microtetraspora*. A total of 134 strains were isolated. This relatively large number of strains isolated can be taken as an indication of the cosmopolitan distribution of this genus. At present no indications of the possible role these micro-organisms might play in the soil are at hand.

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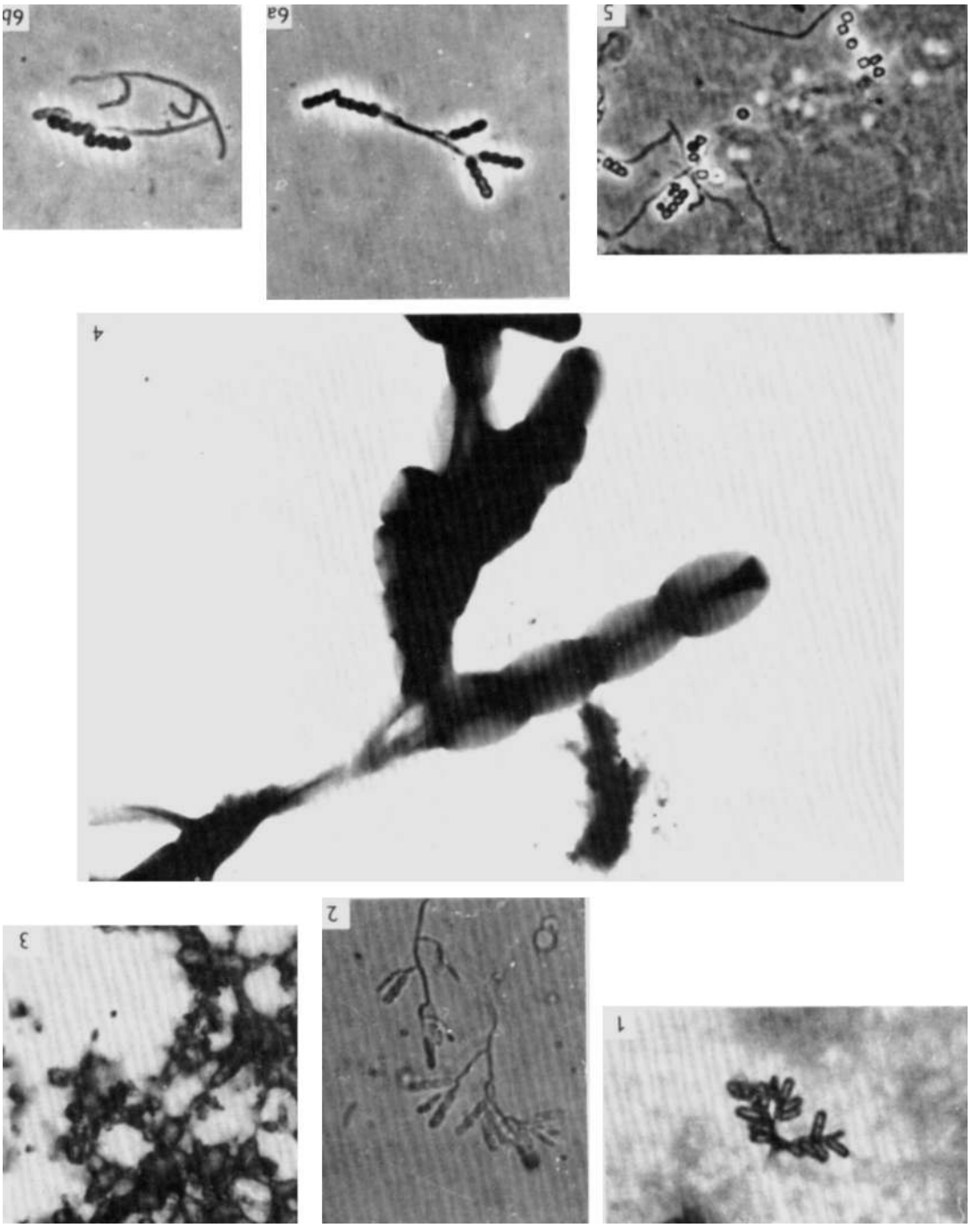

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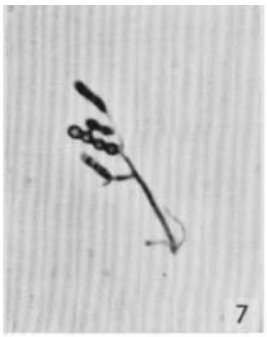
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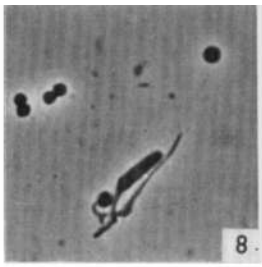
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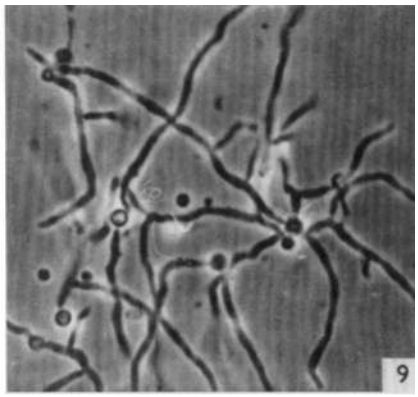
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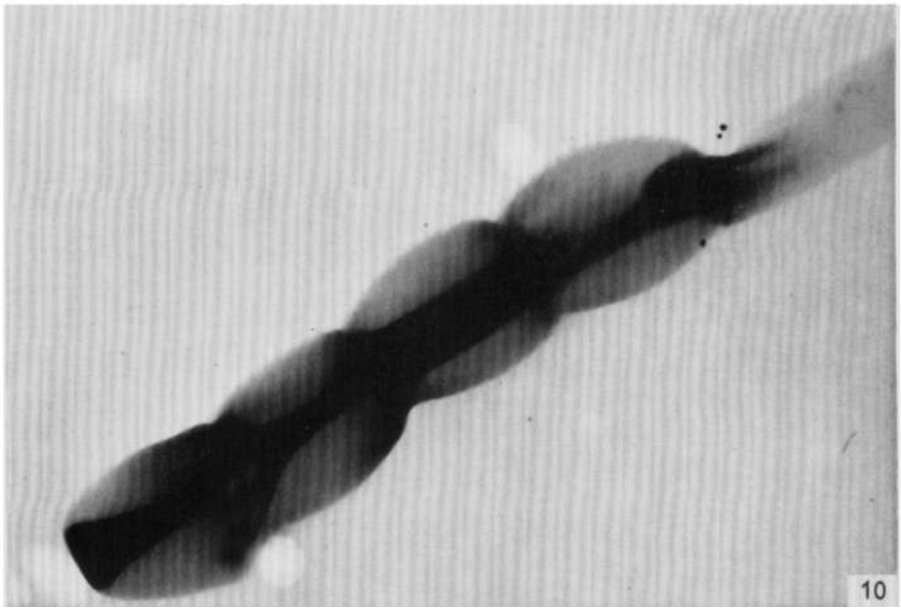
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Microtetraspora glauca* on soil agar plates. Low-power magnification (× 1120) of the aerial mycelium and spore bearing hyphae.

Fig. 2. *M. glauca*; aerial mycelium showing mature spores as well as spore chains in different stages of development (soil plate-direct microscopical examination, × 3500).

Fig. 3. *M. fusca*; spore-fusion into globular masses; Ca malate agar.

Fig. 4. *M. glauca*; electron micrograph of aerial mycelium with sporophores (soil plate, × 10370).

Fig. 5. *M. fucsa*; disrupted chains of spores showing the variable morphology of the still immature spores (× 3500.)

Fig. 6. *M. glauca*; aerial mycelium with fully developed spores (soil plates, × 3500).

PLATE 2

Fig. 7. *Microtetraspora glauca*; different stages of spore formation. Transverse septa are clearly seen (soil plates, × 3500).

Fig. 8. *M. glauca*; formation of transverse septa at the base of the sporophore giving rise to spore formation (× 3500).

Fig. 9. *M. glauca*; spore germination on soil extract agar after 38 hr at 28° (× 3500).

Fig. 10. *M. glauca*; electron micrograph of isolated spore chain (soil plate, × 22,100).